Effect of Allelochemicals of Chinese-fir root extracted by supercritical CO_2 extraction on Chinese fir

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Abstract: Allelochemicals of Chinese-fir root was extracted by technology of supercritical CO_2 extraction under orthogonal experiment design, and it was used to analyze allelopathic activity of Chinese-fir through bioassay of seed germination. The results showed that as to the available rate of allelochemicals, the pressure and temperature of extraction were the most important factors. The allelochemicals of Chinese-fir root extracted by pure CO_2 and ethanol mixed with CO_2 have different allelopathic activities to seed germination, and the allelochemicals extracted by ethanol mixed with CO_2 had stronger inhibitory effects on seed germination than that extracted by pure CO_2.

Keywords: Chinese fir; Autointoxication; Supercritical CO_2 extraction; Bioassay; Seed germination
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Introduction

Allelopathy is one of the most common phenomena in nature. Rice (1984) gave the following definition of allelopathy: "Any direct or indirect harmful or beneficial effect by one plant (including microorganisms) on another through the production of chemical compounds that escape into the environment". Putnam and Tong (1986) stated that chemicals with allelopathic potential were present in virtually all plant tissues, including leaves, flowers, fruits, stems, roots, rhizomes and seeds. Allelochemicals were released by processes as volatilization, root exudation, leaching, and decomposition and so on (Rice 1984; Putnam 1985).

Chinese fir (*Cunninghamia lamcealata* (Lamb.) Hook) spread through seventeen provinces in south China, which is recommended as the most important timber tree species in south China. The field investigation showed that Chinese fir had poor establishment and low productivity, and its growth easily lead to soil degradation. There were many reasons causing soil degradation of Chinese fir man-made forest, concluding the studies of the foregoing researches, autointoxication of Chinese fir was one of the most important reasons (Lin 1999). The effects of allelochemicals of Chinese-fir leaves, stump-roots on seed germination and seedling growth of Chinese fir had been reported in previous studies (Cao 2002a; Huang 2000a). In this study, after extracting the allelochemicals of Chinese-fir root by supercritical CO_2 extraction, we used seed germination bioassay to determine allelopathic activity of allelochemicals to Chinese fir, so as to add new data for Chinese fir autointoxicational study.

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Materials and methods

Collection and treatment of materials

Roots of Chinese fir were collected from the second rotation Chinese fir stand in the vicinity of Fujian Agriculture and Forestry University, Fujian Province, China. The samples were dried at room temperature in a desiccation, and then comminuted by plant disintegrator and stored for further use. Chinese-fir seeds were offered by Fujian Forestry Office.

Extraction of allelochemicals of Chinese-fir root

Allelochemicals of Chinese-fir root were extracted by the method of supercritical CO_2 extraction under orthogonal experiment design. Orthogonal experiment design and the condition of supercritical CO_2 extraction were shown in Table 1. Non-polar-allelochemicals were extracted with pure CO_2, and then ethanol acted as modifier was mixed with CO_2 to extract residue of middle-polar-allelochemicals and polar-allelochemicals, finally concentrated in vacuum at room temperature.

Confection of experimental additives

Allelochemicals extracted by pure CO_2 and CO_2 mixed with ethanol were dissolved by ethanol to obtain solutions with different concentrations of 50, 100, and 200 $mg \cdot kg^{-1}$. Considering ethanol has inhibitory effect on seed germination of Chinese-fir (Liu *et al.* 2001), all of the experimental concentration of additives of ethanol solution were V (ethanol): V (distilled water) =1:100. Control was ethanol with the concentration of 1:100.

Bioassay

The experiment of seed germination of Chinese-fir was conducted in petri dishes at laboratory temperature (27℃) and relative humidity of 60%. Each treatment was replicated three times, with 100 seeds per replication on filter paper. Germination experiment lasted for 10 days. The results of seed germination bioassays were expressed as absolute germination rate, radical length, fresh weight, and dry weight.

Data processing

t-test was used to analysis whether the difference of treatments are remarkable. The absolute germination rate was calculated as:

The absolute germination rate=the total of germination number/ (100—the total of sterile seed) ×100%.

The absolute germination power was calculated as:

The absolute germination power=the amount of germination on the peak day of germination/ (100—the total of sterile seed) ×100%.

Table 1. Available rate of allelochemicals under different conditions of supercritical CO_2 extraction

Component	Weight of materials (g)	Factor				Available rate	
		Pressure (MPa)	Temperature (℃)	Time (h)	CO_2 flux (kg/h)	Pure CO_2 (%)	Ethanol mixed with CO_2 (%)
1	100	30±2	40	2.5	30±3	2.71	0.45
2	100	45±2	40	2.5	50±3	3.12	1.24
3	100	45±2	60	2.5	30±3	3.36	1.02
4	100	30±2	60	2.5	50±3	2.78	1.16
5	100	45±2	40	1.5	30±3	2.76	1.10
6	100	30±2	40	1.5	50±3	2.88	0.87
7	100	30±2	60	1.5	30±3	3.00	0.34
8	100	45±2	60	1.5	50±3	2.98	1.04
	A_1	11.37	11.47	11.97	11.83		
	A_2	12.22	12.12	11.62	11.76	T_A=23.59	
	R_a	0.43	0.33	0.18	0.04		
	B_1	2.82	3.66	3.87	2.91		
	B_2	4.40	3.56	3.35	4.31	T_B=7.22	
	R_b	0.79	0.05	0.26	0.70		

Note: A is available rate of pure CO_2, B is available rate of ethanol mixed with CO_2, R represents extreme difference. 1 represents pressure of extraction 30±2, temperature of extraction 40, time of extraction 2.5, CO_2 flux of extraction 30±3; 2 represents pressure of extraction 45±2, temperature of extraction 60, time of extraction 1.5, CO_2 flux of extraction 50±3.

Results and analysis

The available rates of allelochemicals under different supercritical CO_2 extraction conditions

Different extraction conditions had different effects on the extraction effects of allelochemicals of Chinese-fir roots. Among the extraction factors (Table 1), for the extraction by pure CO_2, pressure and temperature were the most important factors, while for extraction by ethanol mixed with CO_2, pressure and CO_2 flux became the most important factors. Among the three kinds of allelochemicals (non-polar, middle-polar, and polar allelochemicals), the amount of non-polar allelochemicals extracted by pure CO_2 was the largest.

Effect of allelochemicals extracted by pure CO_2 on seed germination

Experimental results showed that the allelochemicals extracted by pure CO_2 under different extraction conditions had different effects on seed germination, and different concentrations of allelochemicals extracted under the same extraction conditions, also had different effects on seed germination (Table 2). The inhibitory effect of the allelochemicals extracted under condition 1, 2, 4 and 7 (Table1) on seed germination was getting weak with the increase of concentration. As for the three treatments in concentration (50, 100, and 200 mg·kg^{-1}), the 100 mg·kg^{-1} allelochemicals extracted under the condition 3 and 6 and 200 mg·kg^{-1} allelochemicals extracted under the conditions 5 and 8, showed the strongest inhibitory effect on seed germination The indices of seed germination showed different responses to the allelochemicals extracted under different extraction conditions and to different concentrations of allelochemicals. For example, for the seeds treated with allelochemicals extracted under condition 1, the absolute germination power, length of plumular root, and length of plumular axis of seed had a increasing trend, but the absolute germination rate, fresh weight and dry weight showed a decreasing trend; for the seeds treated with allelochemicals extracted under condition 2, the absolute germination rate, absolute germination power, plumular root length, plumular axis length and fresh weight were stimulated, but the dry weight was decreased.

Table 2. Effects of allelochemicals extracted by pure CO_2 on germination of Chinese-fir seeds

Component	Treatment /mg·kg^{-1}	Absolute germination rate		Absolute germination power		Plumular root length		Plumular axis length		Fresh weight		Dry weight	
		RI	|t|	RI	|t|	RI	|t|	RI	|t|	RI	|t|	RI	|t|
	50	0.18	2.08	0.38	3.56	0.40	4.88*	0.30	3.76	-0.07	0.81	0.25	2.94
1	100	0.06	2.47	0.27	1.60	0.24	4.34*	0.03	2.43	0.02	0.65	0.19	2.30
	200	-0.03	5.04*	-0.12	6.86*	0.18	0.10	-0.01	0.33	-0.16	5.94*	-0.12	7.77*
	50	0.14	2.95	0.24	1.59	0.29	0.02	0.18	1.58	0.21	11.8**	0.26	2.29
2	100	0.09	3.41	0.23	0.28	0.28	5.61*	0.17	9.92*	-0.12	5.81*	0.23	1.59
	200	0.05	4.08	0.05	0.47	0.24	1.55	0.05	1.45	-0.09	0.41	0.22	3.05
	50	0.18	5.65*	0.20	0.69	0.43	3.55	0.32	2.77	0.10	0.38	0.30	3.05
3	100	0.06	1.50	0.10	1.56	0.01	4.18	0.01	0.82	-0.08	0.23	0.12	3.36
	200	0.17	4.95*	0.11	2.25	0.28	16.1**	0.09	1.60	0.03	3.96	0.17	1.44
	50	0.07	0.72	0.05	0.25	0.36	7.19*	0.38	8.34*	0.16	2.16	0.22	4.72*
4	100	0.08	3.75	0.20	0.53	0.39	0.21	0.36	0.06	0.01	0.47	0.16	2.75
	200	0.04	0.45	0.00	1.49	0.32	3.12	0.31	0.70	-0.16	4.39*	0.14	1.25
	50	0.12	1.05	0.09	3.30	0.28	1.17	0.24	2.56	0.09	0.82	0.30	0.31
5	100	0.02	0.89	-0.26	3.69	0.20	2.76	0.01	7.32*	-0.25	18.4**	0.16	8.00*
	200	0.24	2.29	0.05	1.62	0.37	5.62*	0.23	5.15*	0.31	5.67*	0.26	1.10
	50	0.20	2.45	0.53	10.6**	0.36	5.09*	0.26	27.7**	0.08	2.53	0.31	2.98
6	100	0.08	9.73*	0.21	9.73*	0.30	1.18	0.17	1.32	-0.14	0.99	0.12	2.44
	200	0.14	0.55	0.30	0.01	0.27	1.68	0.16	1.43	0.34	1.77	0.25	0.30
	50	-0.10	0.36	0.06	0.74	0.19	0.38	-0.05	0.11	-0.07	6.13*	0.02	0.23
7	100	0.04	3.11	-0.02	2.04	0.18	2.65	-0.07	2.91	-0.29	1.27	0.09	0.48
	200	-0.19	1.82	-0.12	0.99	0.14	0.99	-0.08	0.80	-0.42	4.11	-0.09	0.00
	50	0.04	2.91	-0.16	1.10	-0.03	2.37	0.17	1.35	-0.11	6.52*	0.00	4.10
8	100	-0.03	1.51	0.40	6.40*	0.15	1.37	-0.03	4.36*	-0.04	0.10	0.00	1.66
	200	-0.05	4.83*	0.23	1.86	0.26	0.24	0.17	2.17	-0.29	6.58*	-0.05	10.4**

Note: The results are the mean value of 3 replicate. Value of RI is the sensitivity index of allelopathy put forward by Williamson: RI=1-C/T(T>C), RI=T/C-1(T≤C). C is the value of control. T is the value of treatment. RI>0 indicates stimulation, RI<0 indicates inhibition. The absolute value of RI stands for the intensity of allelopathy (Williamson, 1988). * P<0.05, * * P<0.01, $T_{0.05}$=4.303, $T_{0.01}$=9.925. The following table is the same.

Effect of allelochemicals extracted by ethanol mixed with CO_2 on seed germination

Allelochemicals extracted by ethanol mixed with CO_2 under different extraction conditions had different effects on seed germination, and different concentration of allelochemicals extracted at the same condition also had different effects (Table 3). For the allelochemicals extracted under the conditions 1, 2, 3, 6 and 7, with the increase of the concentration, their inhibitory effects on seed germination got stronger, and those allelochemicals had the stimulating effect at the low concentrations (50 and 100 mg·kg^{-1}) but inhibiting effect at the high concentration (200 mg·kg^{-1}) on seed germination of Chinese-fir. For the allelochemicals extracted under the conditions 4 and 5, the allelochemical with high concentration of 200 mg·kg^{-1} had stimulating effect on seed germination, compared with low concentrations of 50 and 100 mg·kg^{-1}. In term of indices of seed germination, different indices showed different responses to allelochemicals extracted by different conditions and different concentrations. For example, for the seeds treated with 200 mg·kg^{-1} allelochemicals, extracted under the condition 1, the absolute germination rate, absolute germination power, growth of plumular root, fresh weight and dry weight were inhibited, but the growth of plumular axis was stimulated.

Discussion

DeCandolle (1832) ascribed the problem of "soil sickness" to the toxic exudates produced by crop plants. Since then, much effort has been devoted to the evaluation of root exudates as the source of allelochemicals. McPherson and Thompson (1972) demonstrated that an upland forest of *Q. stellata* and *Q. marilandica* suppressed the growth of understory plants, and that one of the causative factors might be allelochemicals produced by their root and leaves. Frei *et al* (1972) found that the bark of *Q. peduncularis*, *Q. scytophylla* and *Q. magniliaefolia* contained toxic or inhibitory substances inhibiting the growth of orchids. Gliessman (1978) reported that the extracts of green leaves and freshly fallen leaves of *Q. eugeniaefolia* were toxic to cucumber seedlings. Lodhi (1978) reported that *Q. alba*, *Q. borealis* and some other species of plants produced allelochemicals in their leaf litter and soil under the tree. Our results showed that allelochemicals of Chinese-fir root extracted by supercritical CO_2 extraction under different conditions had different effects on the Chinese-fir germination.

It has been demonstrated that allelopathy played an im-

portant role in the dominant growth of some plant species and the formation of plant communities. Many tree species have autointoxication. Cao (1994) reported that aqueous extracts of tea plant stem, leaf, root had inhibitory effect on the germination and seedling growth of tea plant; Du (1999) reported that exudates and extracts of continuously cropped soybean had apparently inhibitory effect on the growth and physiological activities of second-batch seedling; Zhou (1997) reported that tomato had an autotoxidity effect. In term of the study of Chinese-fir autointoxication, Lin (1999) reported that aqueous extracts from surface soil, leaf litter, half decaying leaf litter, fresh leaf, branch, bark and root of Chinese fir had inhibitory effect on seed germination of Chinese fir. Cao (2002b) reported the aqueous extracts of Chinese-fir leaves had inhibitory effect on biomass of Chinese fir. In this study, under different conditions of supercritical CO_2 extraction, the allelochemicals extracted by ethanol mixed with CO_2 had stronger inhibitory effects on seed germination of Chinese fir than that of extracted by pure CO_2.

Allelochemicals are mostly secondary metabolites. These allolochemicals range from simple gases, aliphatic compounds, to complex multiringed aromatic acids including acetic and butyric acids, long chain fatty acids, quinines, simple phenols, phenolic acids derived from cinnamic and benzoic acids (Prasad 1997). As far as the kinds of allelochemicals of Chinese fir were concerned, many researcher reported that phenolic was autointoxicational allelochemicals of Chinese-fir; Chinese fir roots had a higher content of phenolics than that of heart stumps, and edge stumps had the least (Huang 2000a). The phenolic was released during decomposition, and accumulated in the soils around stump-roots (Huang 2000b); Ferulic acid and cinnamic acid had inhibitory effects on seed germination of Chinese fir (Cao 2001). In this study, identification of allelochemicals of Chinese-fir roots will be further studied.

Table 3. Effects of allelochemicals extracted by ethanol mixed with CO_2 on germination of Chinese-fir seeds

Component	Treatment /mg·kg^{-1}	Absolute germination rate		Absolute germination power		Plumular root length		Plumular axis length		Fresh weight		Dry weight	
		RI	|t|	RI	|t|	RI	|t|	RI	|t|	RI	|t|	RI	|t|
	50	0.11	6.13*	0.17	1.45	0.29	5.21*	0.29	5.45*	0.04	0.34	0.03	0.54
1	100	0.08	4.50*	0.20	1.62	0.38	1.24	0.32	0.09	0.00	7.02*	0.03	1.94
	200	-0.02	2.60	-0.08	3.87	-0.32	1.50	0.19	1.65	-0.13	1.39	-0.07	0.72
	50	0.17	2.25	0.37	5.13*	0.33	1.16	0.32	4.54*	-0.02	0.57	0.06	1.00
2	100	0.10	0.38	-0.13	7.98*	0.40	0.46	0.30	0.04	-0.07	0.48	-0.11	1.02
	200	-0.16	0.28	0.03	8.79*	-0.19	3.50	0.42	5.87*	-0.25	4.99*	-0.21	4.26
	50	0.19	18.3**	0.16	0.25	0.43	2.79	0.34	1.55	-0.11	2.26	0.02	1.95
3	100	0.04	3.24	0.09	1.05	0.38	1.90	0.34	3.63	-0.06	0.50	-0.04	0.00
	200	0.06	1.21	0.12	0.28	-0.19	4.47*	-0.15	0.70	-0.07	1.10	-0.06	1.33
	50	-0.05	12.9**	-0.16	4.25	0.30	1.18	0.33	0.38	-0.19	1.03	-0.16	3.61
4	100	-0.06	0.91	-0.14	0.25	0.31	1.11	0.33	1.19	-0.28	1.11	-0.08	1.52
	200	0.07	1.55	-0.15	0.74	-0.24	1.16	0.16	4.26	-0.16	21.9**	-0.03	2.98
	50	-0.13	8.52*	-0.12	1.07	0.23	0.18	0.34	3.01	-0.27	1.78	-0.04	0.80
5	100	-0.14	0.40	0.01	0.81	0.29	2.24	0.30	1.73	-0.25	0.44	-0.18	2.19
	200	-0.02	15.7**	-0.10	1.76	-0.33	1.43	0.27	0.08	-0.08	5.99*	-0.10	5.00*
	50	0.12	2.56	0.29	2.49	0.24	1.19	0.20	2.27	-0.16	0.13	0.16	6.11*
6	100	0.13	3.68	0.16	4.49*	0.29	1.56	0.28	1.32	-0.17	1.08	-0.05	3.46
	200	-0.04	0.43	-0.10	1.70	0.36	0.53	-0.15	0.78	-0.09	0.10	-0.01	0.28
	50	0.15	3.25	0.07	1.45	0.30	3.23	0.30	2.16	0.02	2.90	0.06	2.56
7	100	0.00	0.18	0.14	2.27	0.07	3.40	0.19	6.34*	-0.30	1.29	-0.09	0.00
	200	-0.15	0.11	0.22	1.01	0.20	2.13	0.20	0.48	-0.24	1.19	0.00	1.32
	50	0.00	1.12	0.13	0.24	0.17	7.12**	0.28	20.4**	0.04	1.20	-0.02	1.44
8	100	0.06	8.88**	0.15	0.30	0.37	2.45	0.35	1.42	0.14	1.77	0.16	0.23
	200	0.09	0.06	-0.25	0.79	-0.05	3.54	0.14	4.49*	-0.07	2.08	-0.01	5.00*

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